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For some time microcalcifications associated with breast lesions were considered to represent an epiphomenon, useful only as a radiological indicator of breast carcinoma. However, the data presented here shows that HA crystals are capable of exerting significant biological effects on surrounding cells. In this study we report that HA increases mitogenesis in both normal and malignant mammary cell lines. Particles of latex beads, of similar size and concentration to HA crystals, had no effect on mitogenesis. We have also shown that HA crystals stimulate mitogenesis of quiescent mammary cell lines in a concentration dependent fashion. Our results suggest that exposure to HA crystals can cause a significant increase in $PGE_2$ possibly by induction of COX, which appears to be a critical factor in regulating the mitogenesis of those cells.

These findings support our hypothesis that HA crystals may contribute to breast cancer progression by amplifying the pathological processes surrounding a lesion and underlie the importance of further studies of the pathological potential of microcalcifications consisting of HA crystals in breast oncology.

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Title: Molecular Mechanisms of Calcium Hydroxyapatite·Crystal-induced Mitogenesis in Breast Cancer.
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## Introduction

Radiographic mammary microcalcifications constitute one of the most pertinent markers of both benign and malignant lesions of the breast (Harris *et al.*, 1993). Investigators have shown that calcium in breast tissue is present mainly as calcium phosphates in the crystalline form of hydroxyapatite (HA) with a smaller proportion occurring as oxalates in the form of weddellite crystals (Busing *et al*, 1981; Frappart *et al.*, 1984). The presence of oxalate-type microcalcification appears to be a reliable criterion in favour of the benign nature of the lesion or, at most, of an *in situ* lobular carcinoma (Frappart *et al.*, 1984; Radi *et al.*, 1989). In contrast, calcium HA crystals are found in malignant breast tumours (Busing *et al.*, 1981). Although their diagnostic value is of great importance radiographically, the genesis of these calcifications is unclear. Despite numerous histological ultrastructure studies of HA deposits in breast carcinomas, to date there have been limited investigations of the potential role of these crystals in the propagation and progression of breast cancer.

The diagnosis of cancer is made on the basis of abnormal histologic features and an abnormal pattern of growth. Mitotic cells are more common in malignant tumours than in either benign or normal tissue and the frequency of mitotic cells in a tumour mass is roughly proportional to its rate of growth. The biological effects of HA crystals which may promote breast cancer have been investigated *in vitro* in our laboratory and properties of HA crystals have been observed which emphasise their pathogenic potential. The first is their ability to promote mitogenesis, a characteristic which disrupts the mechanisms that normally control cell division, leading to aggravation of tumour growth. The second is their ability to induce the secretion of proteolytic enzymes such as matrix metalloproteinases (MMPs) leading to degradation of surrounding extracellular matrix components and increase of the cells metastatic potential. We have found that HA crystals enhance mitogenesis in human fibroblasts (HF), MCF-7 breast cancer cells and also normal human mammary epithelial cells (HMEC) as assessed by thymidine incorporation assays. The mechanism of this mitogenic response to HA crystals in breast cancer cells is presently unknown.

It is the goal of this project to further investigate the biological effects of HA crystals in the pathogenesis of breast cancer, specifically, by further characterising breast cancer cells response to HA crystals and elucidating the signalling pathway of HA induced-mitogenesis in breast tumour cell proliferation. This annual summary report covers research for the period July 1$^{st}$, 2000 to June 30$^{th}$, 2001.

## Body

The first specific aim of the proposal as outlined in the technical objective has been addressed, namely the study of the mitogenic response of cells in culture to HA crystals. We examined the mitogenic effect of treating both malignant and normal mammary cells with HA crystals *in vitro*. We looked at the effect of the known mitogens; EGF and FBS on the cells. The mitogenic effect of latex beads, particles of a similar size and concentration to the crystals was also investigated. Mitogenesis was assessed by [$^3$H]thymidine incorporation assays. In all cell lines the addition of

- Attended the 'Mater International Breast Cancer Meeting, Mater Misericordiae Hospital, Dublin, Ireland, February, 2001.
- Maria Morgan in collaboration with Dr. Rob Lewis Daresbury Synchrotron Laboratory, Warrington, UK and Dr. Keith Rodgers, Reader in Crystallography, Cranfield University, Wiltshire, UK, had one days beam-time at the Daresbury Synchrotron Laboratory characterising the calcification present in breast core cut biopsies, May 2001.
- Geraldine McCarthy (principle investigators mentor) was awarded a grant from The Wellcome Trust for a project entitled 'Biological effects of calcium-containing crystals', covering period August 2000-August 2003. The application was based on preliminary work supported by this award.
- Proposal submitted to Bristol-Myers Squibb Foundation, Better Health for Women Foundation initiative in July 2000 based on results generated with the support of this award.
- March 2001, Principal investigator Maria Morgan was awarded a travel fellowship from Enterprise Ireland, International Collaboration Programme 2001 based on preliminary work supported by this award.
- July 2001, Maria Morgan attended the First International Symposium on 'Recent Advances and Future Directions in Breast and Ovarian Cancer' Dublin, Ireland.

## Conclusions

For some time microcalcifications associated with breast lesions were considered to represent an epiphomenon, useful only as a radiological indicator of breast carcinoma. However, the data presented here shows that HA crystals are capable of exerting significant biological effects on surrounding cells. In this study we report that HA increases mitogenesis in both normal and malignant mammary cell lines. Particles of latex beads, of similar size and concentration to HA crystals, had no effect on mitogenesis. We have also shown that HA crystals stimulate mitogenesis of quiescent mammary cell lines in a concentration dependent fashion. Our results suggest that exposure to HA crystals can cause a significant increase in $PGE_2$ possibly by induction of COX, which appears to be a critical factor in regulating the mitogenesis of those cells.

These findings support our hypothesis that HA crystals may contribute to breast cancer progression by amplifying the pathological processes surrounding a lesion and underlie the importance of further studies of the pathological potential of microcalcifications consisting of HA crystals in breast oncology.

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## Figure Legends

Fig. 1 Mitogenic effect of hydroxyapatite crystals on breast cancer cell lines. Confluent, quiescent cultures of MCF-7, Hs578T and HMEC cells were stimulated with HA crystals (18μg/cm$^2$), EGF (0.1ng/ml), FBS (10%), latex beads (18μg/cm$^2$), or left untreated (Ctl). (a) After 24hr cells were pulse labelled with $^3$H-thymidine (1μCi/ml) for 1 hr. Levels of trichloroacetic acid-precipitable $^3$H were determined in quadruplicate, using a liquid scintillation counter. HA caused a statistically significant increase in $^3$H-thymidine uptake over untreated cells ($p \leq 0.05$). All values are given as the mean fold increase over control unstimulated cells ± S.E.M, n=4. (b) Cell counts were performed using a haemocytometer following 48hr stimulation. HA also caused a statistically significant increase in cell numbers ($p \leq 0.05$), n=4. (* = $p \leq 0.05$).

Fig. 2 Effect of varying concentrations of HA on $^3$H thymidine incorporation in breast cancer cell lines. Concentrations of HA varying from 0-42μg/cm$^2$ were added to cell lines; MCF-7, Hs578T and HMEC. All values are given as the mean fold increase over control unstimulated cells ± S.E.M, n=4.

Fig. 3 Inhibition of the mitogenic effect of hydroxyapatite crystals on breast cancer cell lines by bafylomycin $A_1$ (BAF). Confluent, quiescent cultures of MCF-7 cells were stimulated with HA crystals (16μg/cm$^2$) or left untreated (Ctl). 0.1 nM BAF was added to the cells 30 minutes prior to addition of HA crystals. All values are given as the mean fold increase over control unstimulated cells ± S.E.M, n=2.

Fig. 4 (a). Effect of hydroxyapatite (HA) crystals on prostaglandin $E_2$ ($PGE_2$) synthesis in MCF-7 and Hs578T cells. Confluent, quiescent cells were stimulated with HA crystals (18μg/cm$^2$), phorbol myristate acetate (PMA) (1μM), IL-1α (2.5ng/ml), or left untreated (Ctl) for 8hr. All values are given as the mean ± S.E.M., n=3.

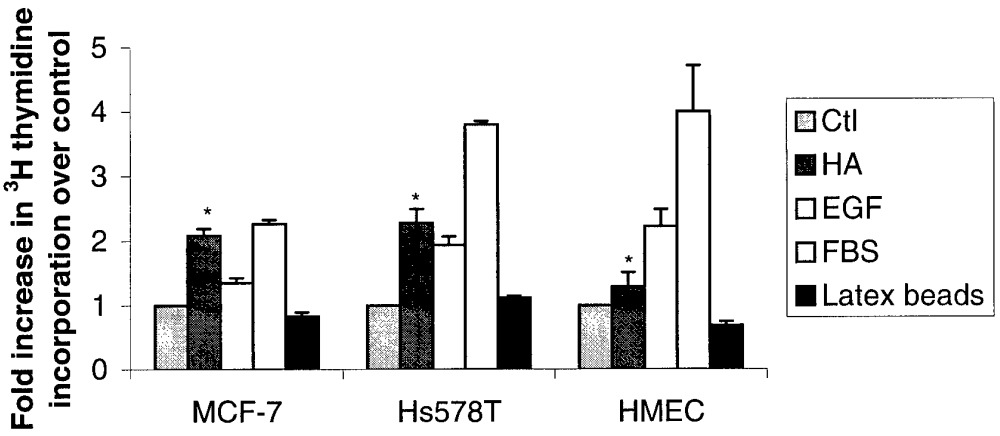
(b). Inhibition of the mitogenic effect of hydroxyapatite crystals on breast cancer cell lines by aspirin. Confluent, quiescent cultures of Hs578T cells were stimulated with HA crystals (16μg/cm$^2$) or left untreated (Ctl). 2mM aspirin was added to the cells 3hours prior to pulse labelling with $^3$H thymidine. All values are given as the mean fold increase over control unstimulated cells ± S.E.M, n=4.

Figure 5 Effect of HA crystals on COX-2 mRNA expression. Doses varying from 0.18 - 180μg/cm$^2$ were added to Hs578T cells and COX–2 and GAPDH (endogenous control) expression analysed by RT-PCR. Latex Beads (LB) particles of a similar size were also used at 18μg/cm$^2$.

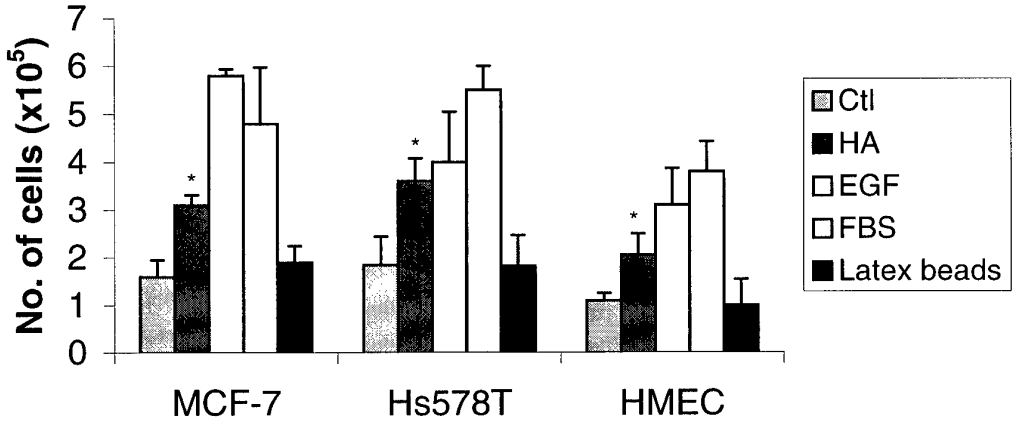
Figure 6. Time course of the induction of COX-2 mRNA expression by HA crystals at 18μg/cm$^2$ analysed by RT-PCR.

## APPENDICES

Includes figures 1-6 and manuscript


Fold increase in $^3$H thymidine incorporation over control
0
1
2
3
4
5
MCF-7
Hs578T
HMEC
Ctl
HA
EGF
FBS
Latex beads

(a)


No. of cells (x10$^5$)
0
1
2
3
4
5
6
7
MCF-7
Hs578T
HMEC
Ctl
HA
EGF
FBS
Latex beads

(b)

Figure 1

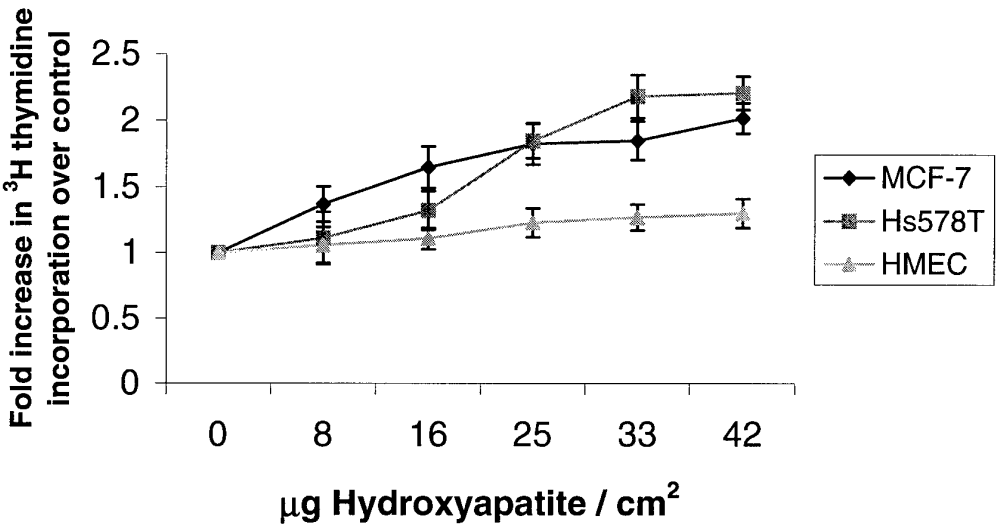

Fold increase in $^3$H thymidine incorporation over control
2.5
2
1.5
1
0.5
0
0
8
16
25
33
42
µg Hydroxyapatite / cm$^2$
MCF-7
Hs578T
HMEC

Figure 2

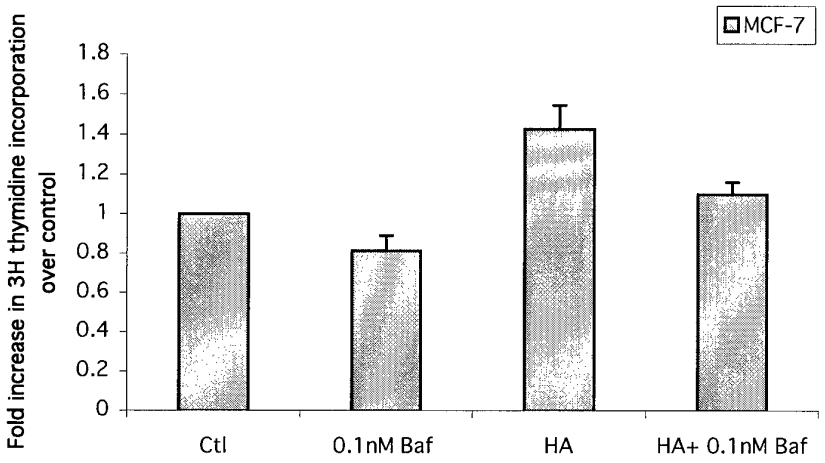
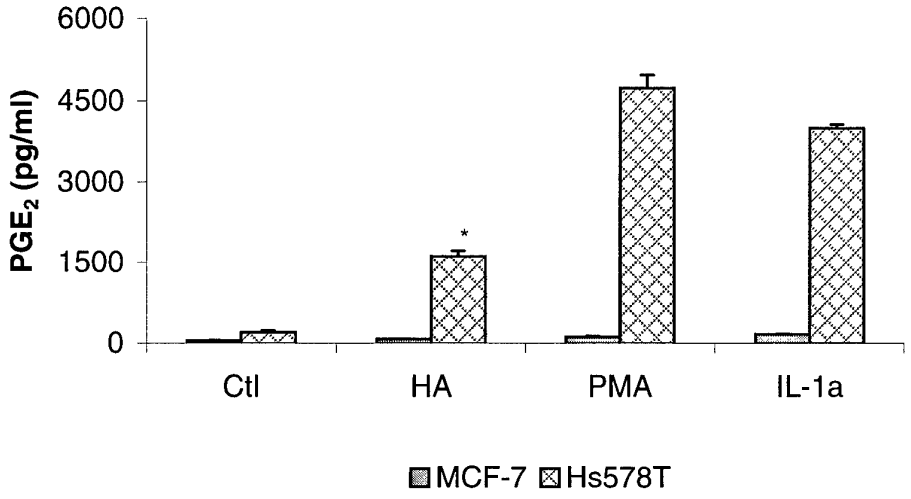
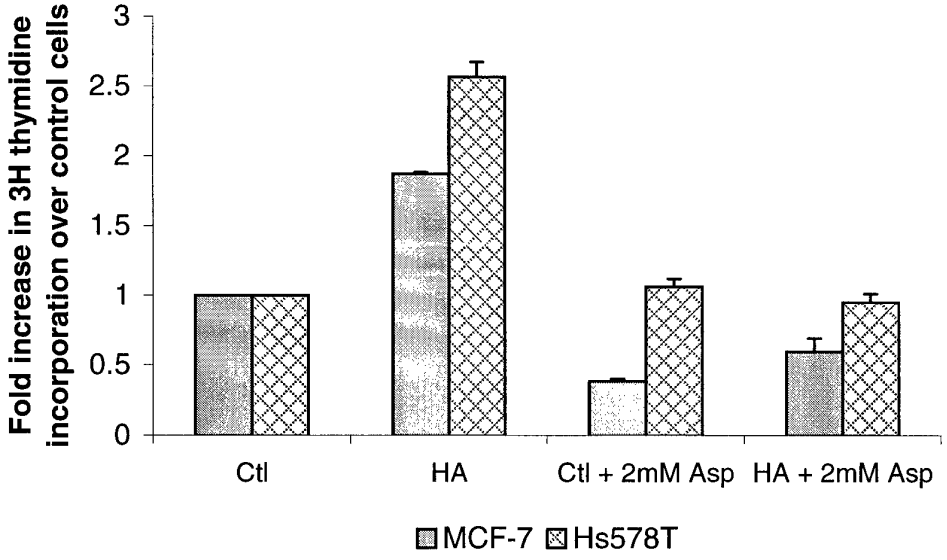

MCF-7
Fold increase in 3H thymidine incorporation over control
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Ctl
0.1nM Baf
HA
HA+ 0.1nM Baf

Figure 3


6000
4500
3000
1500
0
PGE2 (pg/ml)
*
Ctl
HA
PMA
IL-1a
MCF-7
Hs578T

(a)


3
2.5
2
1.5
1
0.5
0
Fold increase in 3H thymidine incorporation over control cells
Ctl
HA
Ctl + 2mM Asp
HA + 2mM Asp
MCF-7
Hs578T

(b)

Figure 4

# EFFECT OF HA ON COX-2 mRNA EXPRESSION IN **Hs578T**

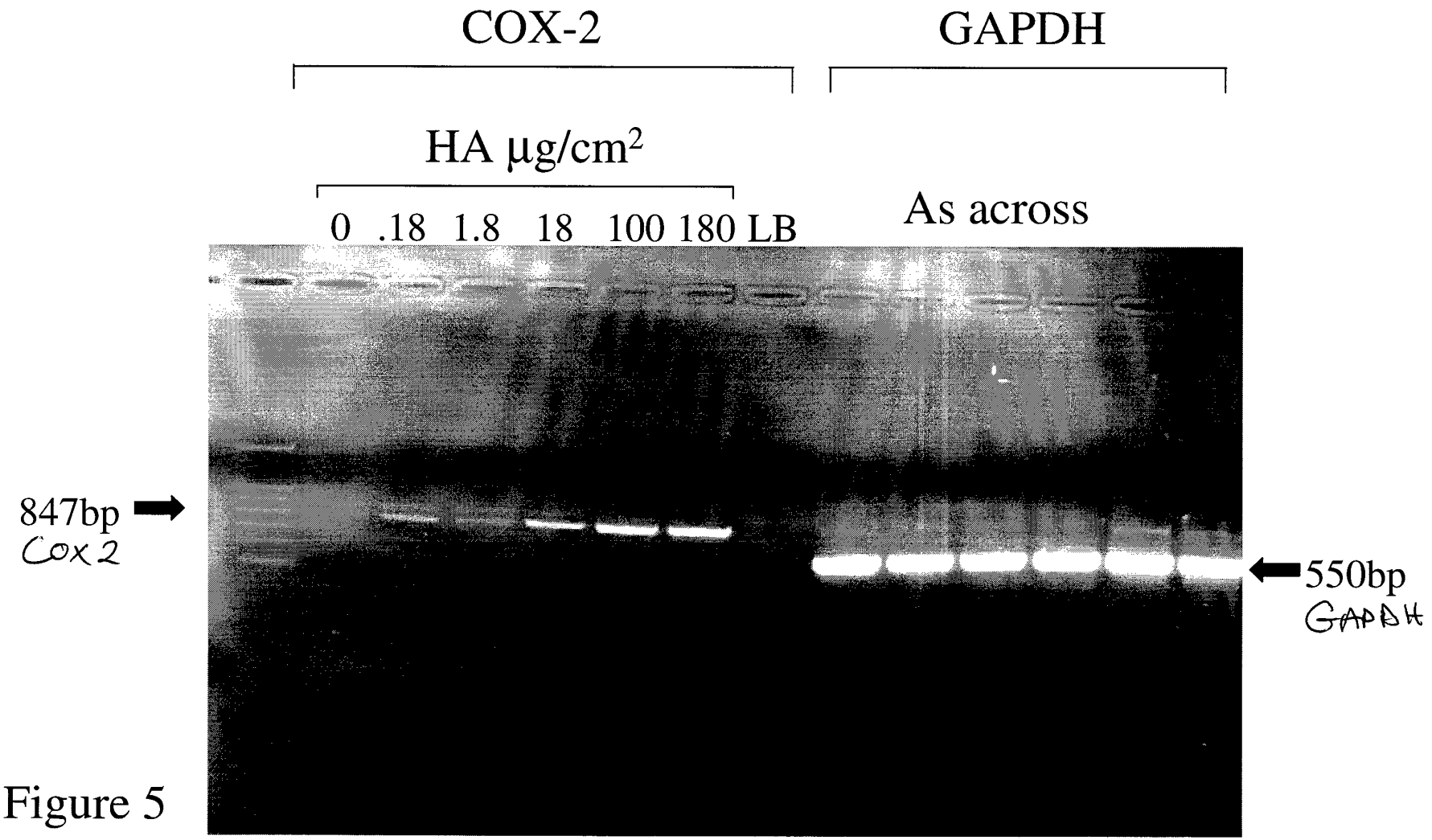



Figure 5

# Time course of HA-induced COX-2 mRNA expression in HS578T

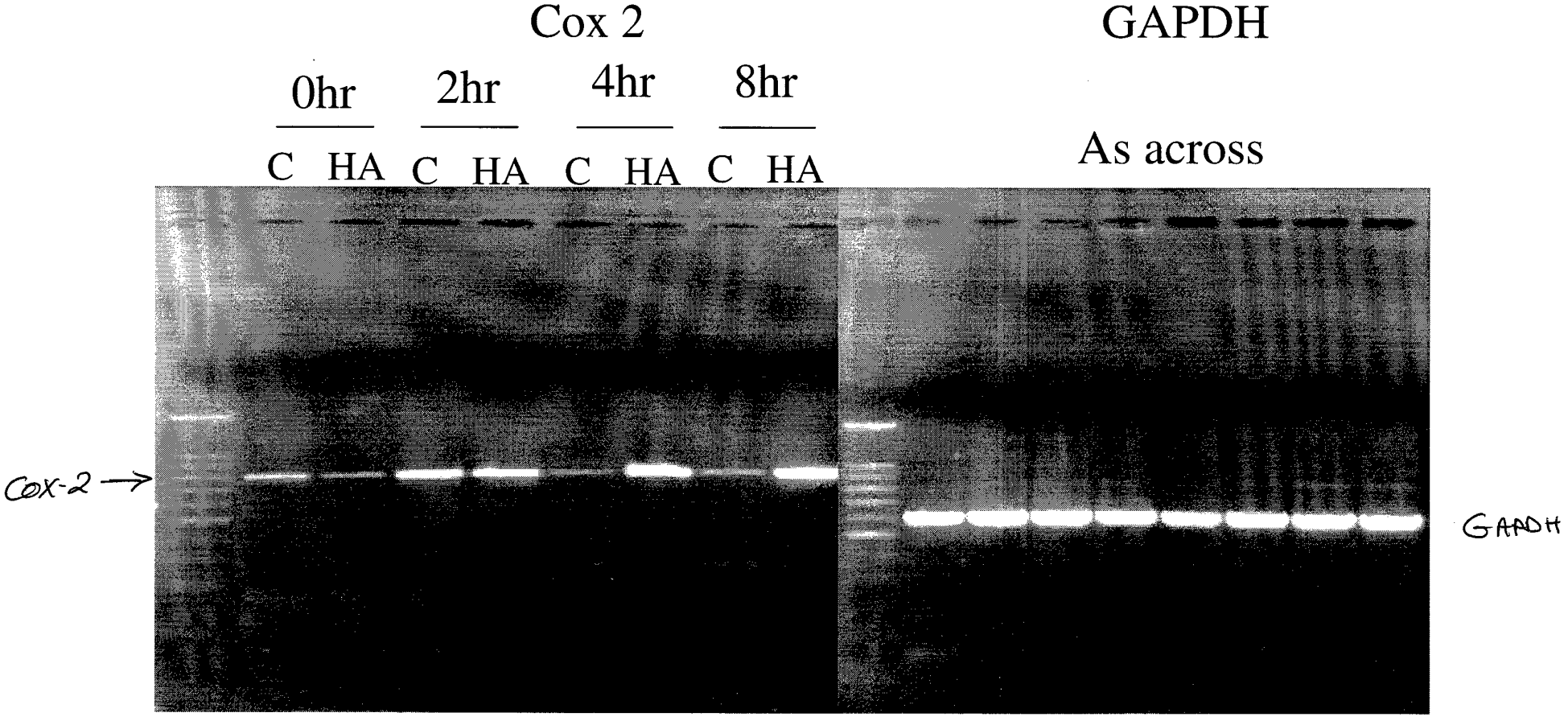



Figure 6

**Calcium Hydroxyapatite Promotes Mitogenesis and Matrix Metalloproteinase Expression in Human Breast Cancer Cell Lines**[1]

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The abbreviations used are: HA, hydroxyapatite; MMP, matrix metalloproteinase; $PGE_2$, prostaglandin $E_2$; HMEC, human mammary epithelial cells; DMEM, Dulbecco's modified Eagles medium; FBS, fetal bovine serum; EGF, epidermal growth factor; PMA, phorbol myristate acetate; IL-1α, interleukin-1α; SDS, sodium dodecyl sulphate; COX, cyclooxygenase.

## ABSTRACT

Radiographic mammary microcalcifications are one of the most pertinent diagnostic markers of breast cancer. Breast tissue calcification in the form of calcium hydroxyapatite is strongly associated with malignant disease. We tested the hypothesis that calcium HA may exert biological effects on surrounding cells, thereby facilitating breast cancer progression. Our findings show that HA crystals enhance mitogenesis in breast cancer cell lines MCF-7 and Hs578T, and also in normal human mammary epithelial cells. HA crystals were also found to upregulate the production of a variety of matrix metalloproteinases, including MMP-2,-9 and –13 in MCF-7 and MMP–9 in human mammary epithelial cell lines. HA crystals were found to greatly augment prostaglandin $E_2$ levels in Hs578T cells and treatment with a cyclooxygenase inhibitor aspirin abrogated the HA-induced mitogenesis. These results suggest that calcium HA crystals may play an active role in amplifying the pathological process involved in breast cancer.

## INTRODUCTION

Radiographic mammary microcalcifications constitute one of the most pertinent markers of both benign and malignant lesions of the breast. Analysis of these microcalcifications by electron microscopy, microprobe analysis and X-ray diffraction has shown that in breast tissue at least two principle types of calcifications can be distinguished according to their structure and chemical composition [1]. Type I microcalcifications are composed of calcium oxalate in the form of weddellite crystals and type II microcalcifications consist of calcium phosphates in the crystalline form of hydroxyapatite (HA), ($Ca_{10}(PO_4)_5(OH)_2$), which is also the basic calcium phosphate found in mature bones and teeth. There is evidence that calcium phosphate and oxalate tend to be associated with different kinds of breast lesions [2]. The presence of oxalate-type microcalcifications appears to be a reliable criterion in favour of the benign nature of the lesion or, at most, of an *in situ* lobular carcinoma and are rarely associated with malignancy [3, 4]. In contrast, the calcifications associated with malignant breast lesions are generally found to be hydroxyapatite [5].

Although their diagnostic value is of great importance radiographically, the genesis of breast calcifications is unclear. The mineralization of breast tissue occurs by deposition of carbonated hydroxyapatite crystals in an extracellular matrix consisting of type I collagen and a variety of noncollagenous proteins. Among these, expression of the bone matrix proteins (bone sialoprotein, osteonectin, osteopontin) and also parathyroid hormone-related protein are believed to play an important role in the initiation and regulation of the deposition of microcalcifications [6]. The luminal calcifications associated with breast lesions appear to be the consequence of an active secretory process by the tumour cells and not solely the result of mineralisation of cellular debris and degenerate tumour cells [7]. The occurrence of microcalcifications

has not been shown to be significantly associated with age or primary tumour size. However, several studies have shown that survival of patients with mammographic microcalcification was significantly shorter than those without [8],[9]. A recent study by Tabar *et al.* also showed that the relative hazard of death from breast cancer was five times higher for tumours with casting-type calcifications than that for circular lesions with no calcifications [10].

The potent biological effects of calcium HA crystals are well recognised in other diseases unrelated to the breast. For example, crystals of basic calcium phosphate, (a term used to describe a mixture of predominantly HA, with small amounts of octacalcium phosphate and tricalcium phosphate) are common in osteoarthritic knee effusions. These crystals clearly potentiate joint damage as their presence and concentration correlates strongly with radiographic evidence and degree of cartilage degeneration [11]. The biological effects of HA crystals which promote articular damage have been well described, and include the induction of synoviocyte mitogenesis, accompanied by upregulation of several members of the matrix metalloproteinase (MMP) family leading to marked synovial proliferation and severe cartilage degeneration [12]. These properties may also be relevant in breast oncology.

There have been numerous histological ultrastructure studies of HA deposits in breast carcinomas. However, despite their potent biological effects in other systems and their association with poorer survival in breast cancer patients, to date there have been no investigations of their potential role in the growth and progression of breast tumours. In the present study, we investigated the pathogenic potential of calcium HA crystals in human breast cell lines by studying their ability to induce mitogenesis, and upregulate prostaglandin $E_2$ ($PGE_2$) and MMP production in MCF-7, Hs578T and normal human mammary epithelial cells (HMEC) cells.

## MATERIALS AND METHODS

**Crystal Synthesis and Preparation:** HA crystals were synthesised by alkaline hydrolysis of brushite as previously described [13]. Mineral prepared by this method has a calcium/phosphate molar ratio of 1.59 and contains predominately calcium hydroxyapatite as shown by Fourier transform infrared spectroscopy. The crystals were sterilised and rendered pyrogen-free by heating at 200°C for 90 min. Crystals were weighed and resuspended by brief sonication in Dulbecco's modified Eagles medium (DMEM).

**Cell Culture:** MCF-7 and Hs578T (American Type Culture Collection, Rockville, MD) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 100mM sodium pyruvate and bovine insulin (4mg/ml). HMEC's and mammary epithelial cell growth media were purchased from Clonetics, Biowhittaker, UK. All cell lines were maintained in a humidified incubator at 37°C with 5% carbon dioxide/95% air.

**Stimulation of Cells in Culture:** Cells were seeded in 24 well plates at $6x10^4$ cells/well and rendered quiescent by incubation in media containing 0.5% FBS for 24 hr. Fresh 0.5% FBS containing media was then added and the cells treated with HA crystals ($18\mu g/cm^2$), epidermal growth factor (EGF) (0.1ng/ml), FBS (10%), phorbol myristate acetate (PMA) (1μM), interleukin-1α (IL-1α) (2.5ng/ml), latex beads (Sigma, St Louis, MO) ($18\mu g/cm^2$), or left untreated (Ctl) for 8hr (for $PGE_2$ assay) or 48 hr (for cell counts and collection of conditioned media and cell lysates).

**[$^3$H]Thymidine Incorporation Assays:** Cells were seeded in 24 well plates at $6x10^4$ cells/well and rendered quiescent by incubation in 0.5% FBS for 24 hr. [$^3$H]Thymidine (1μCi/ml) was added to the wells 23 hr after the addition of HA

crystals (18μg/cm$^2$), EGF (0.1ng/ml), FBS (10%), latex beads (18μg/cm$^2$), or left untreated and pulse labelled for 1 hr. ). Each condition was performed in quadruplicate. To examine the effect of HA-induced $PGE_2$ production on mitogenesis 200mM aspirin was added to cells 3 hours prior to pulse labelling. The cells were then washed and macromolecules were precipitated with 5% trichloroacetic acid solution. Levels of trichloroacetic acid-precipitable $^3H$ were determined in quadruplicate, using a liquid scintillation counter (Wallac 1214 Rackbeta, Turku, Finland).

**Gelatin Zymography:** Sodium dodecyl sulphate (SDS)-polyacrylamide gels were prepared with gelatin (1mg/ml) co-polymerized in the 10% resolving gel and samples containing equal protein concentrations were separated under nondenaturing conditions. Following electrophoresis the gels were washed in 2.5% Triton X-100 for 30 min to remove the SDS and allow the MMPs to renature and then incubated for 24 hr at 37°C in 50mM Tris buffer, pH 7.4, containing 0.15M NaCl and 30mM $CaCl_2$. Gels were stained with Coomassie R-250 and destained with water.

**Western Blots:** Samples containing equal protein concentrations were electrophoresed on 10% SDS-polyacrylamide gels, proteins were electrophoretically transferred to nitrocellulose membranes for 2 hours. Membranes were blocked in 2.5% non-fat dry milk. The membranes were then incubated for 3 hr with 1:500 dilution of primary anti-MMP-13 antibody (R4356, a gift from Peter Mitchell, Pfizer-Central Research Division, Groton, CT). Secondary peroxidase-conjugated anti-rabbit immunoglobulin was used at a dilution of 1/5000. Immunoreactive bands were detected using enhanced chemiluminescence reagents ECL-plus (Amersham Pharmacia Biotech, UK).

**Prostaglandin $E_2$ Immunoassay:** Cells were incubated in Hanks Hepes buffer with 50μM arachidonic acid for 15 minutes and samples collected in duplicate. $PGE_2$

synthesis was measured using a commercially available $PGE_2$ immunoassay from R&D Systems, UK. $PGE_2$ assays were carried out in duplicate 3 times.

**Statistics:** Statistical analysis was performed using the Wilcoxon Rank Sum test.

## RESULTS

### Calcium HA Enhances Mitogenesis.

We examined the mitogenic effect of treating both malignant and normal mammary cells with HA crystals *in vitro*. We looked at the effect of the known mitogens; EGF and FBS on the cells. The mitogenic effect of latex beads, particles of a similar size and concentration to the crystals was also investigated. Mitogenesis was assessed by [$^3$H]thymidine incorporation assays. In all cell lines the addition of 18μg/cm$^2$ HA crystals for 24 hours enhanced mitogenesis above untreated control cells. This increase was statistically significant for all cell lines ($p \leq 0.05$) (Figure 1a). The fold increase over control unstimulated cultures for HA treated cells was 1.29 for HMEC, 2.08 for MCF-7, and 2.28 for Hs578T. The mitogenic effect of EGF another known stimulus of epithelial cells was examined, (2.25 fold increase for HMEC; 1.35 for MCF-7; and 1.94 for Hs578T). The cell lines were routinely grown in 10% FBS, which also had a mitogenic effect (4 fold for HMEC; 2.26 for MCF-7; and 3.8 for Hs578T). Treatment with 18μg/cm$^2$ latex beads had no mitogenic effect on the cell lines.

We also performed cell counts using a haemocytometer to confirm that the increased DNA synthesis was accompanied by an increase in cell number. The mitogenic effect of HA crystals shown by increased thymidine incorporation was confirmed by a statistically significant increase in cell numbers 48 hours following stimulation ($p \leq 0.05$) for all cell lines (Figure 1b). The fold increase in cell numbers

over control of cells treated with HA was; HMEC 1.86; MCF-7 1.93; and Hs578T 1.94. The fold increase over control of cells treated with EGF was; HMEC 2.818; MCF-7 3.62; and Hs578T 2.16. Treatment of the cells with latex beads had no significant effect on cell numbers.

The mitogenic effect of the HA crystals on the cell lines was further characterised by a dose response curve. Concentrations of HA ranging from 0 to 42μg/cm$^2$ were added to the three cell lines. An increase in the mitogenesis of all three cell lines was seen when treated with increasing concentrations of HA (Figure 2).

**Calcium HA Upregulates Matrix Metalloproteinase Production.**

In this study we investigated the effect of HA crystals on MMP-2 and MMP-9 expression using gelatin zymography and MMP-13 expression in MCF-7 cells by Western blotting. Figure 3a shows a zymogram of MCF-7 conditioned media with bands of lytic activity at 92 and 72 kDa representing MMP-9 and –2 respectively. 18μg/cm$^2$ HA crystals caused upregulation of MMP-2 and MMP-9 activity in MCF-7 cells following 48hr stimulation. HA crystals increased MMP-9 and -2 production over control, untreated levels. EGF and FBS also caused induction of MMP expression in these cells. Gelatin zymography also showed upregulation of MMP-9 activity in HMEC cells in response to HA crystals (Figure 3b). EGF and FBS also caused an increase in MMP activity in these cells. In contrast HA stimulation had no effect on MMP-2 or –9 expression in Hs578T cells (data not shown). However, the basal levels of MMP-2 in Hs578T cells were elevated relative to the less invasive MCF-7 cells and Hs578T produced undetectable amounts of MMP-9 by zymography, even after stimulation with EGF or FBS. The lack of induction by HA in the Hs578T

cells may also reflect a change of phenotype associated with the more invasive cell type. MMP-13 protein production was also found to be upregulated in MCF-7s when treated with HA crystals (Figure 3c). MMP-13 expression was not examined in Hs578T.

**Effect of Calcium HA on Prostaglandin $E_2$ Synthesis.**

We examined the effect of HA crystals on $PGE_2$ production in breast cancer cell lines. We found that the biologically aggressive, invasive Hs578T cell line had a higher constitutive level of $PGE_2$ that was approximately 4-fold higher than that observed in the MCF-7 cells (Figure 4a). In addition, HA crystals were found to further augment $PGE_2$ production in Hs578T cells by almost 8-fold, but caused a modest 1.5-fold increase in MCF-7 cells. Similarly, treatment with PMA and IL-1$\alpha$ increased $PGE_2$ production in Hs578T cells by approximately 23-fold and 20-fold respectively, with very little effect on MCF-7 cells. Treatment of the cells with 2mM aspirin, a general cyclooxygenase (COX) inhibitor, blocked the HA-induced increase in mitogenesis of the cells bringing mitogenesis back to levels seen in untreated cells for Hs578T and MCF-7 cells (Figure 4b).

## DISCUSSION

The biological effects of calcium HA crystals on mammary cells were investigated *in vitro* in our laboratory and properties of calcium HA have been observed which emphasise its pathogenic potential. The first is its ability to promote mitogenesis, possibly amplifying the malignant process by leading to aggravation of tumour growth. In this study we report that HA increases mitogenesis in both normal and

malignant mammary cell lines. Particles of latex beads, of similar size and concentration to HA crystals, had no effect on mitogenesis as reported with foreskin fibroblasts [14]. We have also shown that HA crystals stimulate mitogenesis of quiescent mammary cell lines in a concentration dependent fashion. Previously, we have shown that HA crystals stimulate mitogenesis of quiescent cultured human foreskin fibroblasts and adult articular chondrocytes in a concentration dependent fashion [15]. The mechanism of HA crystal-induced activation of human foreskin fibroblasts involves two processes; (1) a fast membrane associated event involving protein kinase C and mitogen activated protein kinase activation, nuclear factor-κB induction and expression of proto-oncogenes *c-fos* and *c-myc* and; (2) the relatively slow endocytosis and intracellular dissolution of the HA crystals raising intracellular calcium causing the activation of a number of calcium-dependent processes leading to cell proliferation [15]. The precise molecular mechanism of HA induced-activation of mammary cells is currently being investigated.

The early proliferative stages of breast cancer are characterised by a continuous basement membrane separating the hyperplastic epithelial cells from the surrounding stroma. Pathologically, the transition from *in situ* to invasive carcinoma is usually accompanied by interruption of the basement membrane caused by an enhanced process of proteolysis contributing to the escape of breast cancer cells into neighbouring tissues, eventually leading to the formation of distant metastases. MMPs are members of a unique family of proteolytic enzymes that can degrade native collagens and other extracellular matrix components [16]. Previous experimental and clinopathological studies have shown good correlations between expression of MMPs and the invasive phenotype of tumour cells [17]. The inducibility of the MMPs by a diverse range of extracellular stimuli has been well documented, including growth

factors, phorbal esters, hormones, steroids, and adhesion molecules [16]. Other studies have demonstrated how inappropriate expression of MMPs can initiate a cascade of events that may represent a coordinated program leading to a phenotypic transformation in mammary epithelial cells [18]. The ability of HA crystals to induce members of the MMP family may reflect a similar cascade of events in our model. The differing effects of HA on MMP expression in the cell lines examined in this study may reflect their contrasting states of differentiation. MCF-7 have an epithelial-like phenotype, are estrogen receptor positive and are weakly invasive, while Hs578T have a more fibroblast-like phenotype, are estrogen receptor negative and are highly invasive. Studies have shown that HA crystals are potent inducers of MMP-1, –3 and –9 in human foreskin fibroblasts and synoviocytes. Cheung and co-workers have recently shown that HA crystals induce MMP-1 expression through an extracellular regulated protein kinase 1 and 2 pathway also involving c-fos/AP-1 and RAS signalling pathways [19].

Elevated levels of $PGE_2$ have been widely reported in many human breast cancers as well as experimental murine mammary tumour models [20]. Several studies with murine mammary tumour cells indicate that $PGE_2$ may have a multifunctional role in controlling growth, metastasis, and the host immune response in breast cancer [21]. Furthermore high levels of $PGE_2$ are often associated with estrogen receptor-negative tumours that exhibit a high metastatic potential [21]. The COX enzymes catalyze the conversion of arachidonic acid to prostaglandins. Calcium-containing crystals have previously been reported to stimulate PG release from cultured mammalian cells accompanied with the release of proteases. Here we have shown that HA can induce $PGE_2$ production in tumour cell lines and that treatment with a COX inhibitor, aspirin can inhibit the HA-induced mitogenic

response. In the present study the contrasting expression of $PGE_2$ in the cell lines MCF-7 and Hs578T may be caused by differential upstream regulation of COX expression. Differential expression and regulation of COX-1 and –2 has been reported in two human breast cancer cell lines (MDA-MB-231 and MCF-7) where COX-2 expression and induction was reported to be influenced by hormone status and metastatic phenotype [22]. Furthermore, we have also recently shown that HA crystals cause induction of COX–2 mRNA and protein in human fibroblasts [23]. Our results suggest that exposure to HA crystals can cause a significant increase in $PGE_2$ possibly by induction of COX, which appears to be a critical factor in regulating the mitogenesis of those cells.

The study presented supports the development of a potentially useful *in vitro* model system to investigate the HA-dependent modulation of mammary epithelial cells and to our knowledge is the first of its kind reported in the literature. The *in vitro* exposure used here is to bath cells with HA crystals while *in vivo* exposure appears to involve cell contact with mineralized deposits [24]. However, consistent with our model is the observation that ultrastructural studies demonstrate the presence of HA crystals in breast cancer cells both in their cytoplasm and associated to their membrane [25].

For some time microcalcifications associated with breast lesions were considered to represent an epiphomenon, useful only as a radiological indicator of breast carcinoma. However, the data presented here shows that HA crystals are capable of exerting significant biological effects on surrounding cells. The induction of MMPs in proliferating tissues could facilitate the clearing of basement membrane and/or connective tissue matrix components to make room for the multiplying cells as they expand. MMP induction and increased $PGE_2$ synthesis may be part of a

programme of gene expression designed for malignant growth. These findings support our hypothesis that HA crystals may contribute to breast cancer progression by amplifying the pathological processes surrounding a lesion and emphasise the importance of further studies of the pathological potential of microcalcifications consisting of HA crystals in breast oncology.

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**Figure Legends**

Fig. 1 Mitogenic effect of hydroxyapatite crystals on breast cancer cell lines. Confluent, quiescent cultures of MCF-7, Hs578T and HMEC cells were stimulated with HA crystals (18μg/cm$^2$), EGF (0.1ng/ml), FBS (10%), latex beads (18μg/cm$^2$), or left untreated (Ctl). (a) After 24hr cells were pulse labelled with $^3$H-thymidine (1μCi/ml) for 1 hr. Levels of trichloroacetic acid-precipitable $^3$H were determined in quadruplicate, using a liquid scintillation counter. HA caused a statistically significant increase in $^3$H-thymidine uptake over untreated cells ($p \leq 0.05$). All values are given as the mean fold increase over control unstimulated cells ± S.E.M, n=4. (b) Cell counts were performed using a haemocytometer following 48hr stimulation. HA also caused a statistically significant increase in cell numbers ($p \leq 0.05$), n=4. (* = $p \leq 0.05$).

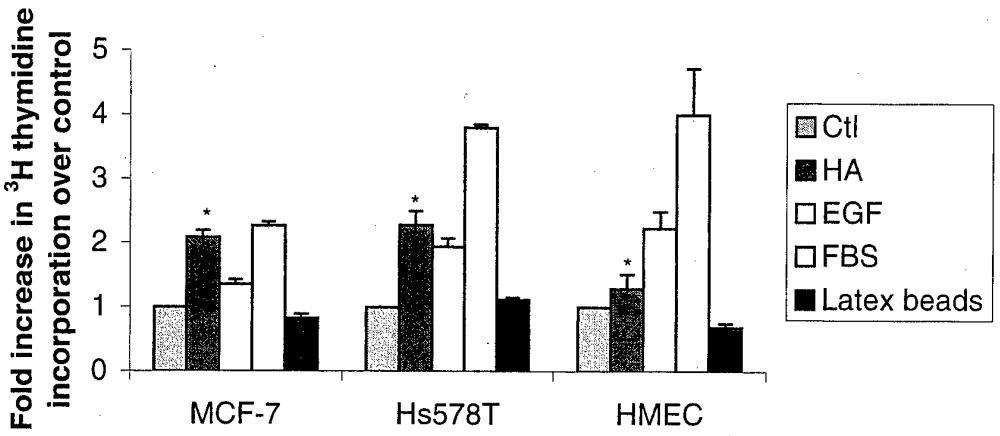
Fig. 2 Effect of varying concentrations of HA on $^3$H thymidine incorporation in breast cancer cell lines. Concentrations of HA varying from 0-42μg/cm$^2$ were added to cell lines; MCF-7, Hs578T and HMEC. All values are given as the mean fold increase over control unstimulated cells ± S.E.M, n=4.

Fig. 3 Hydroxyapatite (HA) crystals induce gelatinase activity in human breast cancer cell lines (a) MCF-7 and (b) HMEC. Confluent, quiescent cells were stimulated with HA crystals (18μg/cm$^2$), EGF (0.1ng/ml), FBS (10%), or left untreated (Ctl) for 48hr. Conditioned media was then collected and analysed by electrophoresis on a 10% polyacrylamide gel containing 1mg/ml gelatin. After overnight incubation at 37°C, gels were stained with Coomassie blue. Digestion of the gelatin substrate is seen as clear band. (c) Effect of HA crystals on MMP-13 protein expression in MCF-7 cells. CM was analysed by Western blot using a polyclonal antibody to MMP-13 (R4356). Gels and blot shown are representative of duplicate experiments.

Fig. 4 (a). Effect of hydroxyapatite (HA) crystals on prostaglandin $E_2$ ($PGE_2$) synthesis in MCF-7 and Hs578T cells. Confluent, quiescent cells were stimulated with HA crystals (18μg/cm$^2$), phorbol myristate acetate (PMA)

(1μM), IL-1α (2.5ng/ml), or left untreated (Ctl) for 8hr. All values are given as the mean ± S.E.M., n=3.

(b). Inhibition of the mitogenic effect of hydroxyapatite crystals on breast cancer cell lines by aspirin. Confluent, quiescent cultures of Hs578T cells were stimulated with HA crystals (16μg/cm$^2$) or left untreated (Ctl). 2mM aspirin was added to the cells 3hours prior to pulse labelling with $^3$H thymidine. All values are given as the mean fold increase over control unstimulated cells ± S.E.M, n=4.


Fold increase in $^3$H thymidine incorporation over control
0
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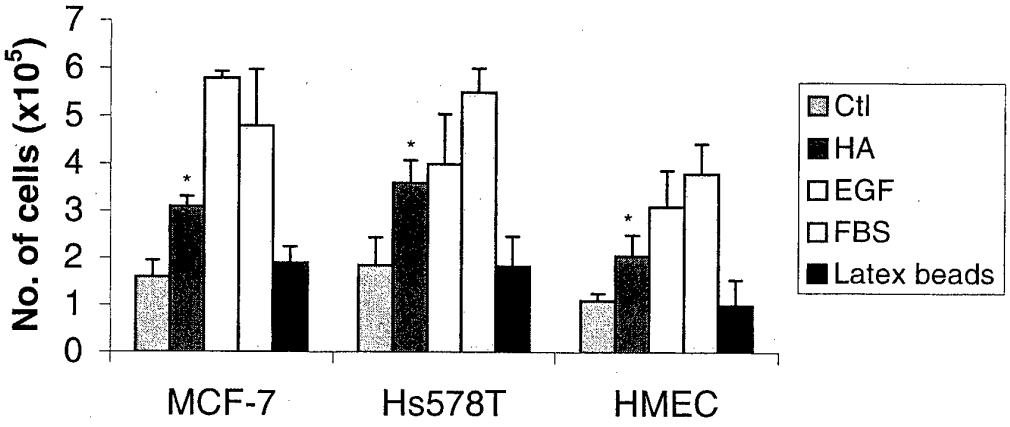
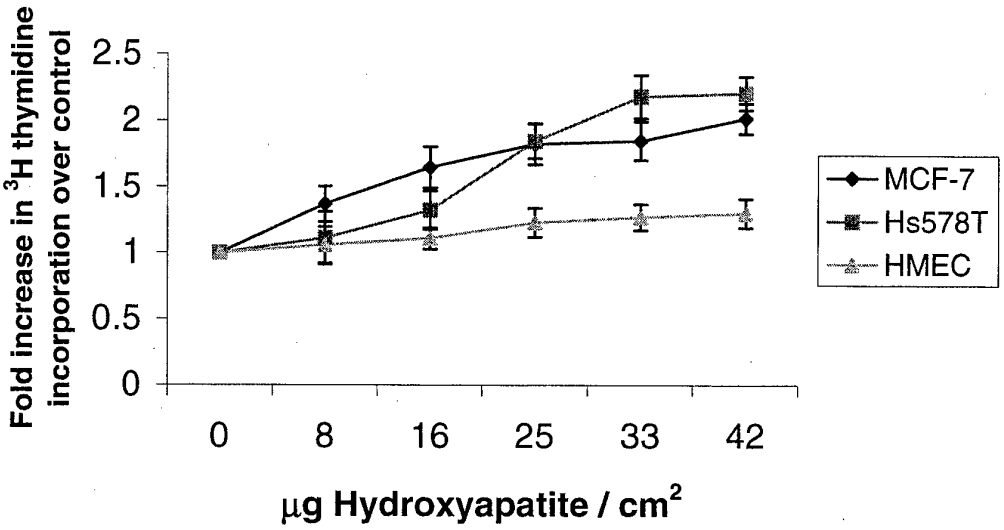
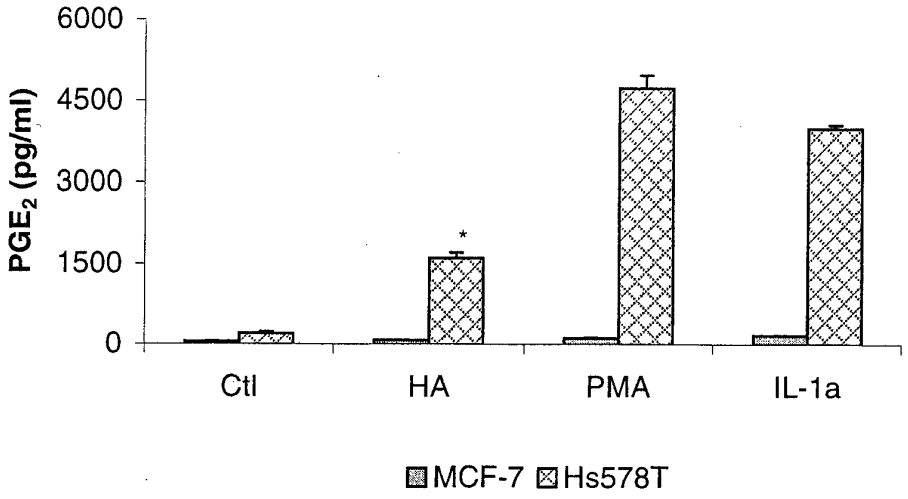
2
3
4
5
MCF-7
Hs578T
HMEC
Ctl
HA
EGF
FBS
Latex beads

(a)

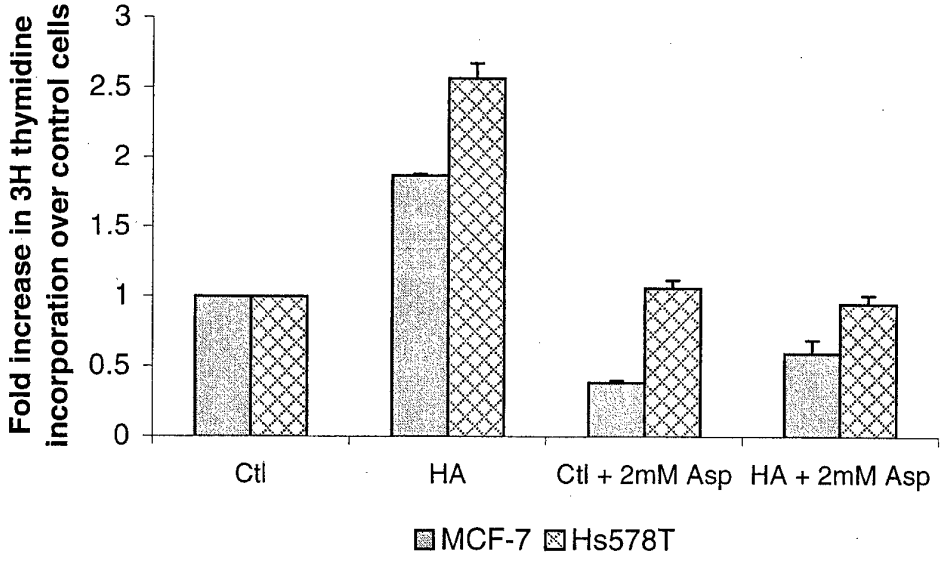

No. of cells (x10$^5$)
0
1
2
3
4
5
6
7
MCF-7
Hs578T
HMEC
Ctl
HA
EGF
FBS
Latex beads

(b)


Fold increase in $^3$H thymidine incorporation over control
2.5
2
1.5
1
0.5
0
0
8
16
25
33
42
μg Hydroxyapatite / cm$^2$
MCF-7
Hs578T
HMEC


6000
4500
3000
1500
0
PGE2 (pg/ml)
*
Ctl
HA
PMA
IL-1a
MCF-7
Hs578T

(a)


3
2.5
2
1.5
1
0.5
0
Fold increase in 3H thymidine incorporation over control cells
Ctl
HA
Ctl + 2mM Asp
HA + 2mM Asp
MCF-7
Hs578T

(b)